

Endothelial Junctional Adhesion Molecule-A Guides Monocytes Into Flow-Dependent Predilection Sites of Atherosclerosis

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Endothelial Junctional Adhesion Molecule-A Guides Monocytes Into Flow-Dependent Predilection Sites of Atherosclerosis

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Background—Junctional adhesion molecule (JAM)-A expressed in endothelial, epithelial, and blood cells can regulate permeability and leukocyte extravasation. Atherosclerosis develops at sites of disturbed flow in large arteries, but the mechanisms guiding inflammatory cells into these predilection sites remain unknown.

Methods and Results—To characterize cell-specific functions of JAM-A in atherosclerosis, we used apolipoprotein E-deficient mice with a somatic or endothelium-specific deficiency in JAM-A and bone marrow chimeras with JAM-A-deficient leukocytes. We show that impaired JAM-A expression in endothelial cells reduced mononuclear cell recruitment into the arterial wall and limited atherosclerotic lesion formation in hyperlipidemic mice. In contrast, JAM-A deficiency in bone marrow cells impeded monocyte de-adhesion, thereby increasing vascular permeability and lesion formation, whereas somatic JAM-A deletion revealed no significant effects. Regions with disturbed flow displayed a focal enrichment and luminal redistribution of endothelial JAM-A and were preferentially protected by its deficiency. The functional expression and redistribution of endothelial JAM-A was increased by oxidized low-density lipoprotein, but confined by atheroprotective laminar flow through an upregulation of microRNA (miR)-145, which repressed JAM-A.

Conclusions—Our data identify endothelial JAM-A as an important effector molecule integrating atherogenic conditions to direct inflammatory cell entry at predilection sites of atherosclerosis. (*Circulation*. 2014;129:66-76.)

Key Words: atherosclerosis ■ cell adhesion molecules ■ endothelial cells ■ imaging, diagnostic ■ microRNAs

Junctional adhesion molecule-A (JAM-A, also known as F11R), a member of the immunoglobulin superfamily, is located in the tight junctions of epithelial and endothelial cells but also expressed by leukocytes and CD34⁺ cells, and exerts a wide variety of functions in cell polarity¹ and barrier function via homophilic interactions,² stem cell adhesion and differentiation,³ leukocyte trafficking, and recruitment.³⁻⁶ In particular, JAM-A mediates leukocyte adhesion and transmigration via heterophilic interactions with the α L β 2 integrin LFA-1.⁷⁻¹⁰ Moreover, dendritic cell trafficking to the lymph nodes was increased in mice with somatic but not endothelial JAM-A deficiency, indicating a specific role in dendritic cell migration.¹¹ Additional findings revealed a crucial role of JAM-A on leukocytes in promoting de-adhesion.¹² In the absence of JAM-A, the directional motility of neutrophils was impaired, leading to incomplete transmigration with neutrophils being stuck between the endothelial cell layer and the basement

membrane.¹² Whereas JAM-A on neutrophils promotes chemotaxis by regulating integrin internalization and recycling,¹³ endothelial JAM-A has been implicated in mediating stimulus-specific neutrophil transmigration.¹⁴ Upon costimulation with tumor necrosis factor (TNF)- α and interferon (IFN)- γ , endothelial JAM-A is redistributed to the luminal surface,^{15,16} and specifically to the site of leukocyte transmigration as part of lateral border recycling compartments.¹⁷

Clinical Perspective on p 76

Compelling evidence has emerged to indicate that JAM-A-mediated leukocyte transmigration plays an important role in inflammatory disease. For example, JAM-A-deficient mice displayed reduced leukocyte infiltration in models of peritonitis, hepatic and myocardial ischemia-reperfusion injury,^{12,18} and in acute lung injury.¹⁹ In models of vascular disease, somatic JAM-A deficiency was shown to protect against

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neointimal hyperplasia after wire-injury of the carotid artery in hyperlipidemic mice.²⁰ Accordingly, increased levels of JAM-A transcripts and protein have been demonstrated in human plaque biopsies and in plaque-prone aortas of mice.²¹ The expression of JAM-A on inflamed and early atherosclerotic endothelium has been implicated in the recruitment of mononuclear cells through an inhibition with soluble JAM-A, suggesting a functional involvement in atherogenesis.⁵

Despite being strategically positioned at junctional entry ports of arterial endothelium, less is known about the cell-specific contributions and net effect of JAM-A in directing mononuclear cell migration during atherogenic recruitment and lesion formation. To dissect the functions of JAM-A in atherosclerosis, we used apolipoprotein E-deficient (*Apoe*^{−/−}) mice with a somatic or endothelial cell-specific deficiency in JAM-A and bone marrow (BM) chimeras with JAM-A-deficient leukocytes. Our data show that endothelial JAM-A promotes lesion formation by integrating effects of hyperlipidemia and disturbed flow, which result in its upregulation, redistribution, and subsequent function in promoting mononuclear cell influx into the artery wall, while limiting reverse transmigration. In contrast, leukocytic JAM-A is required for monocyte de-adhesion and complete transmigration, thereby preventing vascular damage and protecting against atherosclerosis.

Methods

Mouse Models of JAM-A-Deficiency and Plaque Formation

Somatic *JAM-A*^{−/−}*Apoe*^{−/−} were backcrossed for >10 generations into a C57Bl/6 background.²⁰ Congenic *Apoe*^{−/−} mice were used as controls. Chimeric mice with a leukocytic JAM-A deficiency were obtained by reconstituting *Apoe*^{−/−} recipients with *JAM-A*^{−/−}*Apoe*^{−/−} or *JAM-A*^{+/+}*Apoe*^{−/−} BM.²²

A genetic reduction of endothelial JAM-A was achieved by crossing *Apoe*^{−/−} mice with a *loxP*-flanked (floxed) JAM-A gene (*JAM-A*^{fl/fl}) with *VeCad-CreERT2**Apoe*^{+/+} mice to obtain *VeCad-CreERT2**JAM-A*^{fl/fl}*Apoe*^{−/−} mice (C57Bl/6).²³ At an age of 3 weeks, 5 daily intraperitoneal injections of the estradiol analog tamoxifen (0.04 mg/g) dissolved in miglyol 810 were administered to *VeCad-CreERT2*-positive or *VeCad-CreERT2*-negative animals to obtain *eJAM-A*^{−/−}*Apoe*^{+/+} or *eJAM-A*^{+/+}*Apoe*^{−/−} mice, respectively. After 4–6 weeks, genetic depletion of JAM-A was confirmed by FACS analysis, resulting in ≈50% less endothelial JAM-A levels with no difference in leukocytic JAM-A levels compared with control mice. We chose a mouse model with an inducible endothelium-specific Cre-recombinase, because constitutive expression of Cre-recombinase under the control of endothelium-specific promoters (e.g. Tie2/*Tek*, VE-cadherin) can lead to deletion of the floxed gene in hematopoietic cells.^{24,25}

Plaque formation was induced in female littermates (aged 6–8 weeks, n=10–13) by feeding a high-fat diet (HFD, 21% fat, 0.15% cholesterol) for 12 weeks or by partial ligation of the carotid artery, resulting in altered shear stress attributable to reduced and partially retrograde blood flow.²⁶ In brief, *Apoe*^{−/−} mice (n=5) were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg), and left external and internal carotid, and occipital artery were ligated with 6-0 silk sutures, leaving only superior thyroid artery open. Animal experiments were approved by local authorities and complied with German animal protection law (LANUV NRW, Recklinghausen, Germany).

Quantification and Immunohistochemical Analysis of Atherosclerosis

Plaques and lipid depositions in aortic roots and in thoracoabdominal aortas were visualized by Elastica van Gieson or Oil-red-O staining,

respectively, and quantified by computerized image analysis.²² The relative content of macrophages and CD3⁺ T-cells was determined by staining with anti-Mac-2 and anti-CD3, and detection with FITC- and Cy3-conjugated antibodies. Nuclei were visualized with DAPI. Necrotic core areas were quantified in DAPI-stained slices of aortic roots. Stages of atherosclerotic lesions were determined by evaluation of Elastica van Gieson-stained aortic roots.²⁷

Stimulation of Endothelial Cells Under Static or Flow Conditions

Human aortic endothelial cells (HAoECs) without genetic modification, and *JAM-A*^{+/+} *Apoe*^{−/−} or *JAM-A*^{−/−}*Apoe*^{−/−} SV40-large T antigen-immortalized mouse ECs (SVECs) were grown on collagenized glass slides or Bioflux 48-well plates, respectively. HAoECs were treated with low-density lipoprotein (LDL), oxidized LDL (oxLDL; both 10 µg/ml, 16 h), TNF-α and IFN-γ (10 ng/ml and 20 ng/ml, 4 h, respectively), or oxLDL plus JAM-A.Fc (10 µg/ml, 15 min). Mouse ECs were activated for at least 4 h with TNF-α (25 ng/ml). For culture under flow conditions, HAoECs were transferred to 0.4-µm µ-slides (ibidi, Martinsried, Germany). Cell culture was continued under static or flow conditions for 48 h, while shear stress was gradually increased to 5 or 20 dyn/cm² using an ibidi pump system.

In Vitro, Ex Vivo, and In Vivo Monocyte Adhesion and Transmigration Assays

CD14⁺ human monocytes and CD115⁺ mouse monocytes were freshly isolated from human blood or mouse spleens using Monocyte Isolation Kit II or the CD115 mouse MicroBead Kit and were applied on endothelial cells under shear stress (1.5 dyn/cm²) in a laminar flow chamber for up to 10 min and flushed with assay medium for 30 min during time-lapse recordings. Monocytes showing complete, reverse, and incomplete transmigration were counted relative to adherent or total transmigrated monocytes. Human studies were approved by the institutional review board and informed consent was given by the subjects.

For de-adhesion assays, *JAM-A*^{+/+} and *JAM-A*^{−/−} CD115⁺ monocytes were allowed to adhere on TNF-α-stimulated SVEC monolayers or coated intercellular adhesion molecule-1.Fc protein at 0.75 dyn/cm² in µ-slides^{0.4}. Shear stress was gradually increased to 11.5 dyn/cm² and monocytes remaining adherent were counted.

Carotid arteries of *Apoe*^{−/−} mice were explanted, mounted, pressurized at 80 mm Hg, and incubated with FITC-coupled CD31 antibody (5 µg/ml). After stimulation of carotid arteries with TNF-α (10 ng/ml, 3 h), Rhodamine B-stained (1 µg/ml) primary CD115⁺ monocytes with and without JAM-A deficiency (6×10⁵ cells) were perfused in the mounted and pressurized vessel at 0.4 ml/min and visualized using 2-photon laser scanning microscopy (TPLSM).

The right carotid arteries of *eJAM-A*^{−/−} and *eJAM-A*^{+/+} mice were challenged with interleukin-1β (2 µg/ml) carried by a slowly degrading periarterial pluronic gel for 24 h. In addition, Alexa Fluor (AF) 568-coupled anti-CD115 antibody (5 µg/mouse) was administered intravenously. On the next day, anesthetized mice were injected intravenously with anti-CD31 FITC-coupled antibody (35 µg/mouse total) and prepared for in vivo TPLSM by surgically exposing the common carotid artery.²⁸

TPLSM of the Carotid Artery In Vitro and In Vivo

Imaging was performed using a resonant scanning Leica SP5MP (in vivo) or an Olympus FV1000MP with a pulsed Ti:Sapphire Laser tuned at 800 nm and a 20×NA1.00 (Leica) or a 25×NA1.05 (Olympus) water immersion objective.²⁸

Quantification of Immunofluorescence

HAoECs cultured under flow conditions were stained with an anti-JAM-A antibody (10 µg/ml, 2 h, clone M.Ab.F11) followed by secondary antibody staining (anti-mouse immunoglobulin Alexa 594, 1 µg/ml, 1 h) and anti-CD31-FITC-antibodies (10 µg/ml, 2 h,

clone 5.6E). Slides were imaged using a High-Content Imager (BD Pathway 855) with a 20× objective. Per slide, 10 images were taken. Using AttoVision-software, cells were segmented and fluorescence intensity per cell was determined.

Fluorescent Bead Assay

HAoECs were incubated with fluorescent microbeads ($\approx 1 \mu\text{m}$ diameter) coupled to JAM-A antibodies (clone 246) for 30 min. Nuclei were stained with HOECHST 33342. After washing, bright field and fluorescent images were recorded. Bead numbers were related to nuclei count and normalized to binding of immunoglobulin-coupled control beads.

MiR-145 Transfection of HAoECs

HAoECs grown to 80% confluence were transfected with microRNA (miR)-145-3p or miR-145-5p mimics (75 pmol/L) using Lipofectamine 2000. Quantitative (q)PCR analysis of JAM-A mRNA and miR-145 levels was performed after 24 h using established protocols.^{5,29}

Statistical Analysis

Statistical analysis was performed using Prism 6.0 (GraphPad Software). Means were compared between 2 groups by 2-tailed, unpaired, or paired Student *t* test, without or with Welch correction or among more than 2 groups by 1-way ANOVA with Tukey or Bonferroni post test or Kruskal-Wallis test with Dunn post test, as indicated. Differences with $P < 0.05$ were considered as statistically significant.

For more detailed information please see the online-only Data Supplement.

Results

Deficiency in Endothelial JAM-A Protects Against Atherosclerosis

The inhibition of mononuclear cell recruitment by soluble JAM-A has implicated JAM-A in atherogenesis,⁵ however, owing to its pleiotropic functions the overall and cell-specific effects of JAM-A have remained elusive. Notably, somatic JAM-A deletion did not significantly affect atherosclerotic lesion area in the thoracoabdominal aorta and the aortic root of *Apoe*^{-/-} mice fed a HFD for 12 weeks (Figure 1A and 1B). In contrast, reconstitution of *Apoe*^{-/-} mice with *JAM-A*^{-/-}*Apoe*^{-/-} BM (*leuJAM-A*^{-/-}*Apoe*^{-/-}) enhanced diet-induced lesion area in the thoracoabdominal aorta but not in the aortic root, as compared with controls carrying *JAM-A*^{+/+}*Apoe*^{-/-} BM (*leuJAM-A*^{+/+}*Apoe*^{-/-}), indicating an atheroprotective function of JAM-A on BM-derived leukocytes (Figure 1C and 1D). In turn, genetic deficiency in endothelial JAM-A reduced the diet-induced lesion area in the thoracoabdominal aorta and aortic root of tamoxifen-treated *VeCad-CreERT2**JAM-A*^{fl/fl}*Apoe*^{-/-} (*eJAM-A*^{-/-}*Apoe*^{-/-}) mice, as compared with their *Cre*⁻ (*eJAM-A*^{+/+}*Apoe*^{-/-}) littermates (Figure 1E–1H), demonstrating a remarkable role of JAM-A on endothelial cells in promoting atherosclerosis.

Endothelial JAM-A Deficiency Reduces Lesional Infiltration

Immunohistochemistry in the aortic root revealed that the lesional content of Mac-2⁺ macrophages did not differ in somatic *JAM-A*^{-/-}*Apoe*^{-/-} mice versus *JAM-A*^{+/+}*Apoe*^{-/-} controls (Figure 2A). As compared with control chimeras,

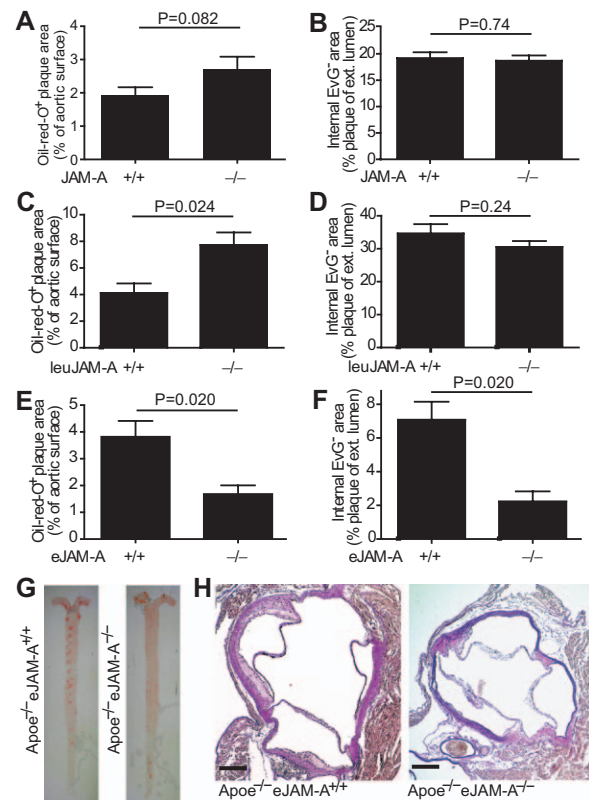


Figure 1. Cell-specific role of junctional adhesion molecule (JAM)-A deficiency in atherosclerosis. *JAM-A*^{+/+}*Apoe*^{-/-} and *JAM-A*^{-/-}*Apoe*^{-/-} mice (A and B), *leuJAM-A*^{+/+}*Apoe*^{-/-} and *leuJAM-A*^{-/-}*Apoe*^{-/-} mice (C and D), *eJAM-A*^{+/+}*Apoe*^{-/-} and *eJAM-A*^{-/-}*Apoe*^{-/-} mice (E and F) were fed a high-fat diet for 12 weeks. Atherosclerotic lesions were quantified in the thoracoabdominal aorta after Oil-red-O staining (A, C, and E) and in the aortic root after Elastica van Gieson staining (B, D, and F). Representative images depict the thoracoabdominal aortas (G) and aortic roots of *eJAM-A*^{+/+}*Apoe*^{-/-} and *eJAM-A*^{-/-}*Apoe*^{-/-} mice (H). Scale bar, 200 μm . Data represent mean \pm SEM (n=10–13), and all *P* values were calculated by 2-tailed *t* test.

JAM-A deficiency in BM-derived cells reduced macrophage content (Figure 2B). Likewise, deficiency in endothelial JAM-A markedly decreased macrophage content, as compared with controls with intact JAM-A (Figure 2C and 2D). The infiltration with CD3⁺ T-cells characterizing early lesion stages was unaffected by somatic deletion of JAM-A but markedly reduced in mice with JAM-A-deficient leukocytes or with an endothelial deficiency in JAM-A (Figure 2E–2G). As a hallmark for advanced lesions, necrotic core size was unaltered by somatic deletion of JAM-A, higher in mice with JAM-A-deficient BM-derived cells, indicative of increased macrophage turnover, and considerably reduced by endothelial deficiency in JAM-A (Figure 2H–2J). To evaluate the rate of lesion development in these mice, we performed a phenotypic classification.²⁷ Whereas *JAM-A*^{-/-}*Apoe*^{-/-} and *leuJAM-A*^{-/-}*Apoe*^{-/-} mice developed lesions that were mainly in an advanced stage, *eJAM-A*^{-/-}*Apoe*^{-/-} mice almost exclusively bore lesions in initial or intermediate stages, displaying an increase in early lesion stages, as compared with controls (Figure 2K). Whereas the preponderance of advanced lesions in chimeric mice is likely related to irradiation, a higher proportion of

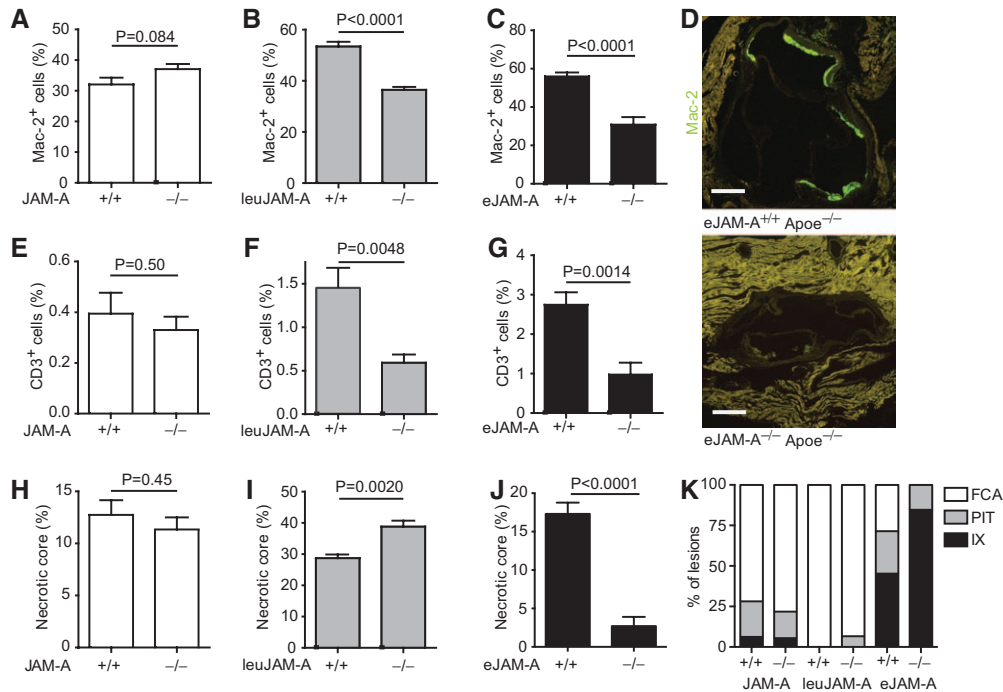


Figure 2. Phenotype of atherosclerotic lesions in junctional adhesion molecule (JAM)-A-deficient mice. The relative content of Mac-2⁺ macrophages (A–D) and CD3⁺ T cells (E–G) in aortic root lesions was analyzed by quantitative immunofluorescence. Representative images of Mac-2–stained aortic roots isolated from *eJAM-A*^{+/+}*Apoe*^{-/-} and *eJAM-A*^{-/-}*Apoe*^{-/-} mice are shown (D). Scale bar, 200 μ m. Necrotic core size in aortic root lesions was quantified after DAPI staining (H–J). *JAM-A*^{+/+}*Apoe*^{-/-} and *JAM-A*^{-/-}*Apoe*^{-/-} mice (A, E, and H), *leuJAM-A*^{+/+}*Apoe*^{-/-} and *leuJAM-A*^{-/-}*Apoe*^{-/-} mice (B, F, and I), and *eJAM-A*^{+/+}*Apoe*^{-/-} and *eJAM-A*^{-/-}*Apoe*^{-/-} mice (C, D, G, and J) were fed a high-fat diet for 12 weeks. The lesions were phenotypically characterized according to the stage (K). FCA indicates fibrous cap atheroma; IX, intimal xanthoma; and PIT, pathologic intimal thickening. Data represent mean \pm SEM (n=10–13), and all *P* values were calculated by 2-tailed *t* test.

less advanced lesion stages in the *eJAM-A*^{fl/fl} lines could be attributable to tamoxifen treatment or residual differences in genetic background. Taken together, cell type–specific effects of leukocyte and endothelial JAM-A deficiency differentially modulate the inflammatory phenotype and stage of atherosclerotic plaques.

Role of Endothelial JAM-A in Arterial Leukocyte Recruitment

We used in vivo, ex vivo and in vitro adhesion and transmigration assays to mechanistically evaluate the role of endothelial and leukocytic JAM-A in arterial monocyte recruitment. Indeed, we found a lower number of wild-type CD115⁺ monocytes that had crossed the endothelial layer of carotid arteries in *eJAM-A*^{-/-} mice after treatment with interleukin-1 β , as compared with *eJAM-A*^{+/+} controls, both during in vivo recording (Figure 3A) and in situ after sacrifice (Figure 3B). Accordingly, the adhesion of wild-type monocytes to JAM-A-deficient endothelial monolayers stimulated with TNF- α /IFN- γ was unaltered but their transmigration underneath JAM-A-deficient endothelial monolayers was markedly reduced, compared with JAM-A-bearing endothelial cells (Figure 3C and 3D). The decreased transmigration of monocytes across JAM-A-deficient endothelial cells might also be explained by an increase in reverse transmigration, which was only observed when JAM-A^{+/+} leukocytes transmigrated across JAM-A-deficient endothelial cells (Figure 3E).

Leukocytic JAM-A Deficiency Impairs De-Adhesion and Endothelial Barrier Function

As compared with wild-type monocytes, *JAM-A*^{-/-} mouse monocytes showed a marked increase in firm adhesion to ex vivo perfused wild-type carotid arteries (Figure 3G) and to *JAM-A*^{+/+} endothelial cells in a recruitment assay under flow conditions in vitro (Figure 3C). This was accompanied by reduced transmigration of *JAM-A*^{-/-} monocytes (Figure 3D), which may be attributable to a defect in de-adhesion.¹² *JAM-A*^{-/-} monocytes showed a higher rate of incomplete transmigration (defined as cells that had entered endothelial junctions without exiting, thus being entrapped) across both JAM-A-expressing or JAM-A-deficient endothelial cells (Figure 3F), explaining the overall decrease in complete transmigration (Figure 3D). To confirm defective de-adhesion of *JAM-A*^{-/-} monocytes, we performed assays under variable flow conditions. Compared with *JAM-A*^{+/+} controls, adherent *JAM-A*^{-/-} monocytes were more resistant to shear stress and detachment induced by gradually increasing flow rates both on stimulated SVEC monolayers (Figure 3H) and the β 2 integrin ligand intercellular adhesion molecule-1.Fc (Figure 3I). This clearly indicates that de-adhesion was impaired in *JAM-A*^{-/-} monocytes and that this effect was likely mediated by increased activity of β 2 integrins.

Because compromised endothelial integrity is a hallmark of early atherosclerosis,³⁰ we investigated the permeability of the carotid artery wall of *Apoe*^{-/-} mice deficient in leukocytic and endothelial JAM-A fed with or without HFD for 6

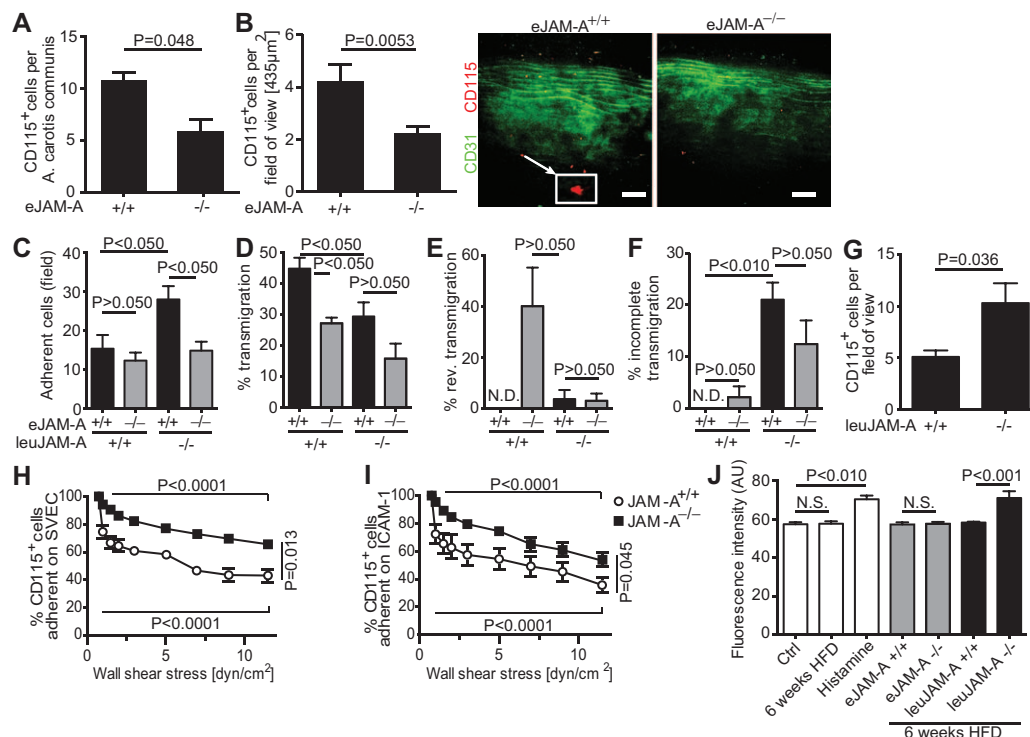


Figure 3. Cell-specific effects of junctional adhesion molecule (JAM)-A on transmigration and permeability. **A** and **B**, Numbers of CD115⁺ cells located in the vessel wall of common carotid arteries after incubation with interleukin (IL)-1 β in a perivascular pluronic gel for 24 h were analyzed by in vivo (**A**) and post mortem in situ (**B**) 2-photon laser scanning microscopy (TPLSM) in *eJAM-A*^{+/+} (n=3) and *eJAM-A*^{-/-} mice (n=7–9). Representative images depict CD115⁺ monocytes (red; inset shows magnification) migrated into sub-endothelial regions, CD31⁺ endothelium and elastin autofluorescence in green (**B**, right). Scale bar, 40 μ m. **C–F**, Stable adhesion (**C**) completed (**D**), reverse (**E**), and incomplete (**F**) transendothelial migration of *leuJAM-A*^{+/+} or *leuJAM-A*^{-/-} monocytes perfused on tumor necrosis factor (TNF)- α /interferon (IFN)- γ -treated *eJAM-A*^{+/+} (black bars) or *eJAM-A*^{-/-} endothelial cells (grey bars) at 1.5 dyn/cm² was analyzed by video microscopy (n=3–5). **G**, Adhesion of primary *JAM-A*^{+/+}*Apoe*^{-/-} (n=6) and *JAM-A*^{-/-}*Apoe*^{-/-} (n=7) CD115⁺ mouse monocytes was determined using TPLSM after ex vivo perfusion of TNF- α -activated carotid arteries (*JAM-A*^{+/+}*Apoe*^{-/-}) pressurized at 80 mm Hg. **H** and **I**, De-adhesion of primary *JAM-A*^{+/+} and *JAM-A*^{-/-} CD115⁺ mouse monocytes was assessed by detachment of adherent cells at gradually increasing flow rates on TNF- α -activated SV40-large T antigen-immortalized mouse EC (SVEC) monolayers (**H**) or intercellular adhesion molecule-1.Fc (**I**; n=12 each). **J**, Permeability was assessed by extravasation of 70-kDa FITC-labeled dextran using 2-photon laser scanning microscopy (TPLSM) in carotid arteries ex vivo with or without (ctrl) histamine-treatment or after 6 weeks of high-fat diet in *Apoe*^{-/-} mice (open bars), with or without deficiency of endothelial (n=5, grey bars) or leukocyte (n=5, black bars) JAM-A. Data represent mean \pm SEM, and *P* values were calculated by 1-way ANOVA with Bonferroni (**C–F**) or Tukey (**H–J**) multiple comparison test or 2-tailed *t* test (**A**, **B**, **G–I**).

weeks. As assessed by ex vivo TPLSM, the uptake of fluorescent dextran into the arterial media was not affected by HFD or endothelial JAM-A deficiency but increased by histamine-treatment or leukocytic JAM-A deficiency (Figure 3J). Altered vascular permeability in *leuJAM-A*^{-/-}*Apoe*^{-/-} mice may reflect the damage inflicted by impaired de-adhesion or transmigration of *leuJAM-A*^{-/-} leukocytes and may explain increased lesion formation.

Focal Redistribution of Endothelial JAM-A in Lesion-Prone Areas

Analysis of atherosclerotic arteries and plaque tissue revealed an increase in JAM-A expression.^{5,21} We used ex vivo 2-photon laser scanning microscopy (TPLSM) in unfixed and intact carotid arteries of *Apoe*^{-/-} mice to label JAM-A and to determine its distribution in plaque-prone vessels as a potential mechanism underlying atherogenic leukocyte recruitment (Figure I in the online-only Data Supplement). The undiseased common part of the carotid artery explanted from 1 year-old *Apoe*^{-/-} mice on normal chow showed an organized mesh-like junctional pattern of JAM-A expression in

luminal endothelial cells (Figure 4A, left and Movie I in the online-only Data Supplement). In contrast, segments near the bifurcation, which are prone to lesion formation owing to disturbed flow, displayed an apico-luminal redistribution and focal concentration of JAM-A away from endothelial cell junctions (Figure 4A, middle and right). The middle panel shows the transition zone between undiseased segments with a normal junctional JAM-A pattern (left part) and focal enrichment of JAM-A in the shoulder region of a lesion (right part). To assess the propensity of JAM-A for early focal redistribution during lesion formation, we performed costaining with PECAM-1 (CD31) as another junctional protein expressed in endothelial cells (Figure 4B and Figure II in the online-only Data Supplement). In carotid arteries of *Apoe*^{-/-} mice fed a HFD for 6 weeks, typical examples of costaining for JAM-A and CD31 in the undiseased common part and the plaque-prone areas near the bifurcation are shown (Figure 4B, left and middle). Using image analysis, we determined the pseudo-overlap of JAM-A and CD31 expression in undiseased areas for normalization (Figure 4B, right). The bifurcation prone to nascent atherosclerotic lesion formation

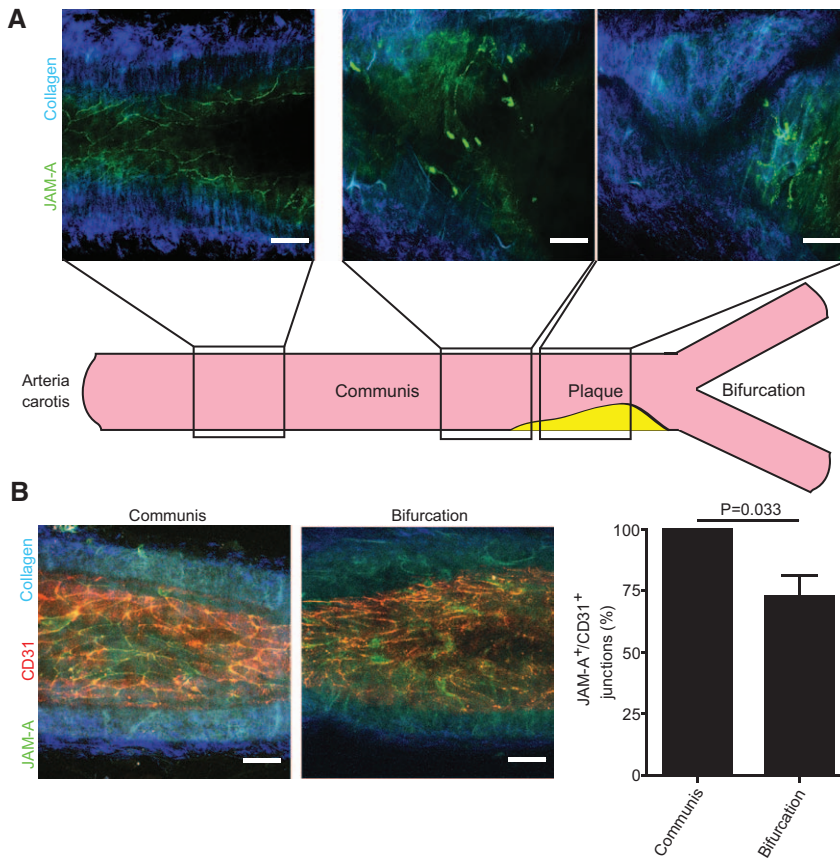


Figure 4. Arterial localization of junctional adhesion molecule (JAM)-A during atherogenesis. **A**, Visualization of JAM-A (green) and collagen (blue, SHG) in an explanted carotid artery of a 1-year-old *ApoE*^{-/-} mouse fed normal chow. An organized junctional pattern of JAM-A staining is evident in the healthy part of the carotid artery (left), which changes into a focally enriched pattern in the shoulder (middle) and core (right) regions of the plaque. Images are total projections of z-stacks. A schematic drawing (lower part) depicts where images were taken. Scale bar, 40 μ m. **B**, Costaining (left and middle) and semiquantitative analysis (right) of CD31 (red) and JAM-A (green) in the carotid artery of an *ApoE*^{-/-} mouse fed a high-fat diet for 7 weeks revealed a decreased JAM-A/CD31 pseudo-overlap in the endothelial junctions at the diseased bifurcation, compared with the common part. Scale bar, 40 μ m. Data represents mean \pm SEM (n=5), and P value was calculated by 2-tailed t test.

revealed a decrease in junctional colocalization of JAM-A and CD31 ($72.8 \pm 8.4\%$ of control, n=5, $P < 0.05$), implying a preferential redistribution of JAM-A.

Oxidized LDL Increases Functional JAM-A Expression

To elucidate whether the focal accumulations of endothelial JAM-A in atherosclerotic lesions are caused by redistribution or increased *de novo* synthesis, we performed JAM-A antibody-coupled fluorescent bead assays, real-time PCR, and Western blot analysis. Similar to costimulation with TNF- α and IFN- γ , treatment with oxLDL but not with native LDL increased specific surface binding of anti-JAM-A microbeads to aortic endothelial cell monolayers (Figure 5A and 5B). Blocking *de novo* protein synthesis with cycloheximide partially yet nonsignificantly reduced JAM-A surface levels, suggesting that both increased expression and redistribution contributed to the up-regulation of endothelial surface JAM-A. Treatment with oxLDL but not native LDL or TNF- α , dose-dependently increased JAM-A transcript and protein expression in HAoECs, reaching a plateau at 2.5 μ g/ml of oxLDL (Figure 5C). To link JAM-A expression to atherogenic recruitment, we performed a transmigration assay using classical CD14⁺ monocytes and HAoECs under shear flow conditions (Figure 5D). Similar to costimulation with TNF- α /IFN- γ , treatment with oxLDL but not native LDL caused an increase in monocyte transmigration. Notably, blocking endothelial JAM-A with soluble JAM-A.Fc reduced the oxLDL-induced increase in transmigration to levels seen in untreated

HAoECs, indicating that the effect of oxLDL on transmigration is mediated by JAM-A.

The Expression and Localization of JAM-A Is Governed by Shear Flow and miR145

Because the increased susceptibility to lesion formation in areas near bifurcations or branching points is related to altered flow conditions (eg, low or oscillatory flow), we tested whether such aberrant flow conditions as induced by partial ligation of the carotid artery of *ApoE*^{-/-} mice affects the localization of JAM-A. Indeed, costaining for JAM-A and CD31 revealed a discontinuous pattern of JAM-A and redistribution from the junctions in the common part of partially ligated carotid arteries, as seen in the bifurcation of mice with established lesions (Figure 6A and Figure II in the online-only Data Supplement). The redistribution of JAM-A was evident both in the common part and in the bifurcation, as early as 2 weeks after ligation (Figure 6A). The pseudo-overlap of JAM-A with CD31 was markedly reduced in carotid arteries after 2 weeks and this reduction sustained for at least 8 weeks after partial ligation (Figure 6B), indicating that JAM-A is preferentially redistributed from its junctional expression pattern under conditions of disturbed flow. Conversely, the control of JAM-A might be linked to atheroprotective laminar flow conditions. Hence, we assessed JAM-A expression in HAoECs cultured under static or flow conditions, revealing an inverse correlation of JAM-A expression with laminar shear flow (Figure 6C and 6D). To correlate this effect with a JAM-A-dependent local predilection for atherosclerosis,

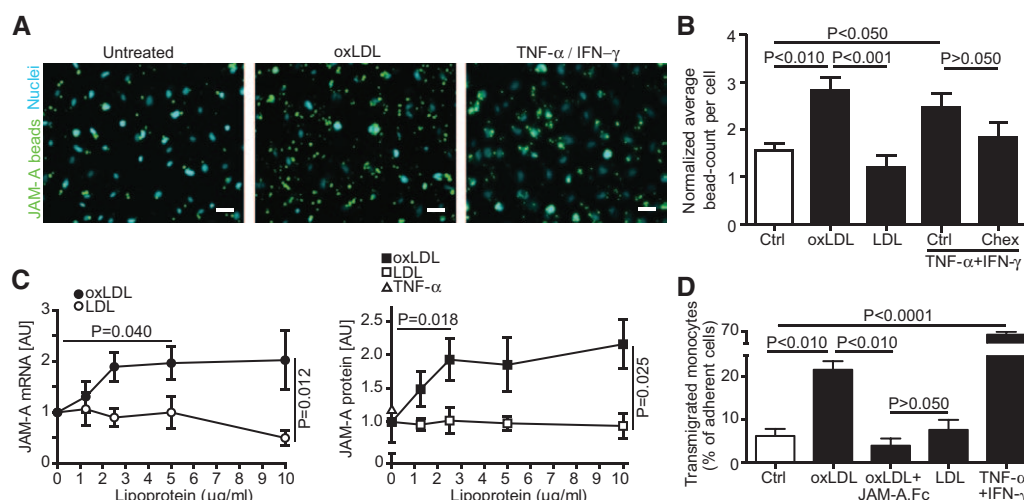


Figure 5. Expression and localization of junctional adhesion molecule (JAM)-A under atherogenic conditions. **A** and **B**, Human aortic endothelial cells (HAoECs) were treated with or without low-density lipoprotein (LDL), oxidized low-density lipoprotein (oxLDL; 16 h), tumor necrosis factor (TNF)- α /interferon (IFN)- γ (4 h), or cycloheximide (Chex, 1 h), before incubation with fluorescent microbeads coupled to JAM-A antibodies (green). After washing, representative images were taken (**A**), and beads were counted (**B**) and normalized to the number of endothelial cells (blue). Scale bar, 40 μ m. Counts were corrected for unspecific binding using beads carrying isotype control. **C**, HAoECs were treated with LDL (open symbols) or oxLDL (solid symbols) for 16 h at indicated concentrations. Quantitative real-time PCR normalized to GAPDH (left, $n=3$) and Western blot analyses normalized to Ponceau staining (right, $n=5$) were performed. **D**, Transendothelial migration of CD14⁺ monocytes across HAoECs treated with or without (open bar) LDL, TNF- α /IFN- γ , or oxLDL and with or without blocking JAM-A.Fc fusion protein (black bars) under flow conditions. Transmigrated cells were counted and normalized to the total number of adherent cells. Data represent mean \pm SEM of 3 independent experiments. P values were calculated by 1-way ANOVA with Tukey multiple comparison test (**B** and **D**) or 2-tailed t test (**C**).

in vivo analysis confirmed that the reduction of atherosclerotic lesion formation in the descending aorta and the aortic arch between *eJAM-A*^{+/+}*Apoe*^{-/-} and *eJAM-A*^{-/-}*Apoe*^{-/-} mice was more prominent at regions of low/oscillatory shear stress (eg, in the inner curvature of the arch or the descending aorta with branching points), as compared with regions with high/laminar shear flow (eg, the outer curvature of the arch and the abdominal aorta; Figure 6E and 6F).

Recent evidence has linked the expression of miR-145 in endothelial cells under shear flow conditions to the mechanosensitive transcription factor Krüppel-like factor 2.^{31,32} Indeed, culture of HAoECs under shear flow conditions increased the expression of miR-145, as compared with static conditions (Figure 6G). Notably, an analysis of miR target sequences revealed that the gene of JAM-A harbors at least 2 seed sequences for miR-145-3p and miR-145-5p (see Figure III in the online-only Data Supplement). To investigate whether JAM-A expression is regulated by a miR-145-dependent mechanism, we transfected HAoECs with miR-145-3p and miR-145-5p mimics. Notably, both the miR-145-3p and miR-145-5p strands reduced the copy number of JAM-A mRNA in HAoECs (Figure 6H). These findings suggest that miR-145 is a flow-dependent regulator of JAM-A expression, which may protect against plaque formation by attenuating JAM-A mRNA levels under flow conditions. Taken together, endothelial JAM-A levels might serve to functionally integrate atherogenic risk factors, such as modified LDL and disturbed flow conditions.

Discussion

Here we have unraveled the cell-specific effects and mechanisms exerted by JAM-A in diet-induced atherosclerosis,

and the overall and context-dependent consequence of its genetic deficiency. Previous studies have reported differential or even contradictory effects of somatic and endothelial JAM-A deficiency in various models of tissue injury and inflammation.^{14,17,33,34} This apparent inconsistency may be attributable to the use of heterogeneous models affecting different vascular beds. We used diet-induced atherosclerotic lesion formation and arterial inflammation in *Apoe*^{-/-} mice and found that endothelial JAM-A promoted lesion formation and mononuclear cell infiltration. This effect was mediated by an elevated accessibility of JAM-A (through upregulation and redistribution) and its function in guiding monocyte recruitment, which was stimulated under atherogenic conditions. The expression and luminal enrichment of JAM-A was exacerbated by disturbed flow conditions (eg, reduced shear flow), as encountered at predilection sites, whereas laminar shear flow induced miR-145 to repress JAM-A expression, in a mechanism that may contribute to limiting the susceptibility to atherosclerosis. Targeting endothelial JAM-A may thus serve as a feasible option to protect against atherogenic inflammation.

In contrast, we demonstrate that JAM-A deficiency in BM-derived leukocytes aggravates atherosclerotic lesion formation. Dendritic cells deficient in JAM-A display higher migratory capacity in lymphoid organs but not in blood vessels,¹¹ whereas JAM-A-deficient polymorphonuclear cells show reduced diapedesis in peritonitis and ischemia-reperfusion injury, related to impaired de-adhesion and polarized motility.¹² We found that JAM-A-deficient monocytes indeed display defects in de-adhesion and complete transendothelial migration, likely a result of persistently increased activity of β 2 integrins. Consequently, monocytes become entrapped

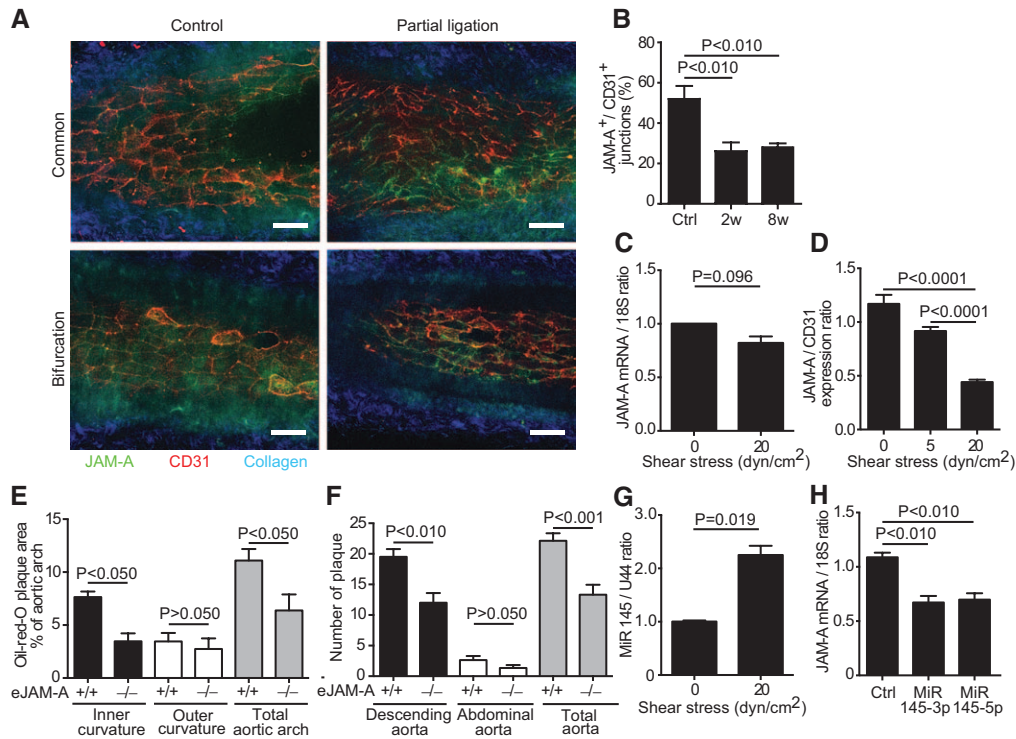


Figure 6. Expression and localization of junctional adhesion molecule (JAM)-A during altered flow conditions. **A**, Visualization of JAM-A (green), CD31 (red), and collagen (blue) in control (left) and treated carotid arteries (right) at the common part and the bifurcation, 2 weeks after partial ligation. Images are total projections of z-stacks. Scale bar, 40 μ m. **B**, Quantification of the percentage of junctions staining positive for both JAM-A and CD31 in controls and carotid arteries, 2 and 8 weeks after partial ligation (n=4–6). **C** and **D**, Expression of JAM-A mRNA relative to 18S housekeeper, as analyzed by qRT-PCR (**C**), and expression of JAM-A protein relative to CD31, as quantified by immunofluorescence (**D**) in human aortic endothelial cells (HAoECs) cultured under static or flow conditions, as indicated (n=19–32). **E** and **F**, Quantification of Oil-red-O⁺ lesion surface in areas of low/oscillatory shear flow (inner curvature), high/laminar shear flow (outer curvature), or the total aortic arch (**E**), and number of plaques in the descending, abdominal and total aorta (**F**) of eJAM-A^{+/+}Apoe^{-/-} and eJAM-A^{-/-}Apoe^{-/-} mice fed a high-fat diet for 12 weeks (n=10–13). **G** and **H**, Expression of miR-145 relative to U44 housekeeper in HAoECs cultured under static or flow conditions (**G**) and expression of JAM-A mRNA relative to 18S housekeeper in HAoECs transfected with miR-145-3p, miR-145-5p mimics or control (**H**), as analyzed by qRT-PCR (n=3–4). Data represent mean \pm SEM, and P values were calculated by 1-way ANOVA with Tukey multiple comparison (**B**, **E**, **F**, and **H**), Kruskal-Wallis with Dunn's post-test (**D**), or 2-tailed t test (**C** and **G**).

between the endothelial cell layer and the basement membrane and may thereby cause vascular damage. Our data thus indicate that the presence of leukocytic JAM-A may limit atherogenic damage by enabling physiological de-adhesion and migration of mononuclear cells.

No significant alterations of atherosclerotic lesion formation were found in mice with somatic JAM-A deletion, pointing towards an intermediate phenotype between the atherogenic influence of endothelial JAM-A and the protective functions of leukocytic JAM-A. Notably, the reduction in Mac-2⁺ and CD3⁺ cell content in mice with either endothelial or leukocytic deficiency in JAM-A was not recapitulated in JAM-A^{-/-} mice, where no difference could be observed. This may be attributable to counteracting effects of JAM-A deficiency in cells other than endothelial and BM-derived cells (e.g. smooth muscle cells, pericytes, or non-BM-derived resident macrophages). The intermediate phenotype was also reflected in the classification of lesion stages. In general, irradiation for BM reconstitution appeared to accelerate the development of more advanced stage plaques, whereas the deficiency in endothelial JAM-A delayed lesion progression and clearly favored a retention in early lesion stages.

We used various assays to assess the capacity of JAM-A-deficient leukocytes or endothelial cells to support recruitment in vivo, ex vivo and in vitro and we provide novel in vitro evidence that a deficiency in endothelial JAM-A might also allow for monocytes to reappear on the monolayer by passively permitting reverse migration, contributing to the overall decrease in complete transmigration. This retentive control exerted by JAM-A complements a reverse gate-keeper function revealed for JAM-C in a study in which blocking JAM-C reduced the number of monocytes in inflammatory tissue by increasing reverse transmigration.³⁵ Accordingly, a hesitant or reverse transmigration behavior has been identified for neutrophils during inflammation after ischemia-reperfusion injury in vivo, which was characterized by lower expression of JAM-C at endothelial junctions, enhanced by blockade or genetic deletion of JAM-C³⁶, and implicated in systemic dissemination of inflammation.

Mechanistically, the exacerbation of atherosclerotic lesion formation by endothelial JAM-A appears to be attributable to its redistribution from the intercellular junctions to the luminal surface, and to an upregulated expression under atherogenic conditions, thus increasing the availability of luminal JAM-A. Previous work has addressed the redistribution of

endothelial JAM-A, showing that JAM-A is relocalized to the apical surface in a cytokine-dependent manner under static or flow conditions *in vitro*.^{15–17,34} We extend these findings by unveiling a focal upregulation and redistribution of JAM-A expression in endothelial cells of carotid arteries under atherogenic conditions of hyperlipidemia. The increased luminal JAM-A availability could mediate enhanced recruitment and higher content of macrophages and T-cells in atherosclerotic plaques of hyperlipidemic mice. *In vitro* experiments confirmed that oxidized LDL increased expression of JAM-A and its redistribution to the apical surface.

Our data challenge the intuitive assumption that knocking-out a protein that is crucially involved in junctional barrier function would cause a nonspecific influx of leukocytes. Rather, the evidence presented in this study refutes a role of JAM-A in limiting transendothelial migration and points towards a specific role of JAM-A in leukocyte recruitment in the context of atherosclerosis. First, genetic deficiency in endothelial JAM-A decreased infiltration of plaques with macrophages and T cells and reduced luminal monocyte extravasation in carotid arteries *in vivo*. Second, redistribution of JAM-A induced by oxLDL facilitated the transmigration of monocytes.

The increase in expression and focal redistribution of JAM-A in response to atherogenic stimulation with oxLDL reveals a striking similarity to JAM-C, a related JAM family member, which displays increased expression and redistribution to the apical surface after oxLDL treatment.³⁷ Like for JAM-A, JAM-C predominantly localized to interendothelial contacts under quiescent conditions, oxLDL induced a disorganized JAM-C localization that was no longer restricted to interendothelial junctions, and JAM-C supported both adhesion and transmigration upon oxLDL stimulation.³⁷

The upregulation and luminal redistribution of JAM-A is enhanced by aberrant shear flow conditions (e.g. after partial ligation or at predilection sites), whereas laminar shear flow appears to exert beneficial effects on JAM-A, limiting the susceptibility to atherogenesis. Accordingly, the reduction of atherosclerotic lesion formation in the aortic arch was more prominent in regions of low shear rather than high shear (e.g. the inner curvature of the arch and branching points of the descending aorta). Of note, Krüppel-like factor 2–transduced or shear stress–stimulated human endothelial cells have been found to be enriched in miR-143/145, providing a mechanism for atheroprotective communication with neighboring SMCs via miR-145-containing microvesicles.³² Our data confirmed that shear flow induces an increase in miR-145 expression in arterial endothelial cells, which is highly relevant and extends findings identifying JAM-A as a miR-145-target in tumor cells.³⁸ The overexpression of miR-145 suppressed the expression of JAM-A in endothelial cells, which was mirrored by the flow-dependent reduction in JAM-A expression. Thus, miR-145 is a regulator of JAM-A in the setting of arterial remodeling.

Notably, platelet endothelial adhesion molecule-1 (PECAM-1), another Ig family adhesion molecule expressed on platelets, leukocytes, and endothelial cell junctions, has recently been found to regulate atherosclerotic plaque formation in a cell- and site-specific manner.³⁹ In contrast to JAM-A,

PECAM-1 on BM cells was proatherogenic irrespective of the hemodynamic environment, whereas PECAM-1, like JAM-A, reduced lesion formation in areas of disturbed flow but was atheroprotective in high shear regions by mechanisms yet to be elucidated.

In the context of arterial denudation injury, the neointimal content of macrophages was reduced in mice with a somatic JAM-A deletion. This was likely attributable to a reduced JAM-A–dependent deposition of the chemokine CCL5 by platelets on the luminal surface of injured arteries.²⁰ Moreover, the phenotype of regenerating endothelial cells after wire-injury may differ, benefiting from increased JAM-A expression and its function in endothelial cell migration and shear resistance.⁴⁰

Our study identifies the differential and cell type–specific contribution of JAM-A in atherosclerosis, namely a crucial involvement of endothelial JAM-A in atherogenic leukocyte recruitment in response to modified lipids and at predilection sites with disturbed flow. Increased expression and focal redistribution of JAM-A controlled by miR-145 establish a novel set of mechanisms to explain the higher susceptibility to atherosclerosis in regions with disturbed shear flow. Endothelial JAM-A may serve as a suitable marker for molecular imaging of early stages of lesion formation and may be developed as a therapeutic target to treat atherosclerosis.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Atherosclerotic lesions develop in the larger arteries at sites of disturbed flow, but the mechanisms behind this observation still remain poorly defined. In this study, Schmitt and colleagues investigated the cell-specific roles of junctional adhesion molecule A (JAM-A) in a mouse model of atherosclerosis. Through the use of 2-photon laser scanning microscopy on intact mouse arteries, the authors discovered that under aberrant flow conditions and hyperlipidemia, the cellular localization of endothelial JAM-A changes from a defined junctional to a focal apical pattern, thereby facilitating the recruitment of monocytes to the developing plaque. In vitro studies revealed that the expression of JAM-A was decreased by micro RNA 145, a micro RNA that is preferentially expressed under laminar flow conditions. These results highlight endothelial JAM-A as a proinflammatory factor that links aberrant flow conditions to inflammatory cell recruitment. These findings might be used to design novel and specific molecular imaging probes for the noninvasive detection of early endothelial dysfunction and for the therapeutic targeting of JAM-A in vascular inflammatory disease.